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**METHOD OF ASSAYING PYRROLE-CONTAINING  
BIOLOGICAL COMPOUNDS**

1           Cross-Reference to Related Application

2  
3           This application is a divisional of co-pending  
4 U.S. Application No. 09/970,328, filed October 2,  
5 2001, which is a continuation-in-part of U.S.  
6 Application No. 09/679,141, filed October 3, 2000  
7 (now abandoned), the disclosures of which are  
8 incorporated herein by reference.

9  
10                   **BACKGROUND OF THE INVENTION**

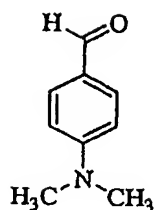
11                   Field of the Invention

12           This invention relates to methods of assaying  
13 pyrrole-containing biological compounds and chemical  
14 compositions that can be used in such methods. More  
15 specifically, it relates to a method for detecting  
16 pyrrole-containing molecules that are markers of  
17 particular disease states.

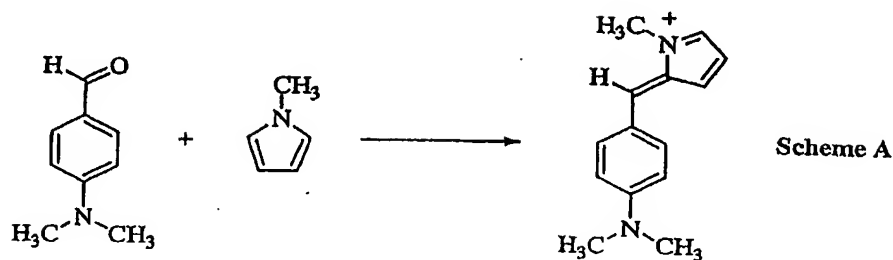
18  
19                   Description of Related Art

20           Erich's reagent, or *p*-dimethylaminobenzaldehyde  
21 (1), is a molecule that can react with pyrroles and  
22 indoles to form a chromogenic compound.

1



2 See G. Lombard and V. Dowell, *J. Clin. Microbiol.*



3 (1983) 18:609-613. The mechanism of action is  
 4 typically described as an electrophilic attack on the  
 5  $\alpha$ -carbon atom of a pyrrole. This attack forms a  
 6 highly conjugated cation that absorbs light in the  
 7 visible spectrum. Such a mechanism is graphically  
 8 represented in Scheme A above.

9  
 10 The reaction of Ehrlich's reagent with certain  
 11 compounds has been discussed. For instance, Iyer  
 12 reported a pyrrole is formed when LGE<sub>2</sub> is reacted  
 13 with proteins. See Iyer et al., *J. Org. Chem.* (1994)  
 14 59:6038-6043. When the pyrrole was contacted with  
 15 Ehrlich's reagent in the presence of BF<sub>3</sub>·OEt<sub>2</sub>, a blue-  
 16 green chromophore was produced. The chromophore was  
 17 identified as a pyrrolic electrophilic substitution  
 18 product.

1 Lombard reported the reaction between Ehrlich's  
2 reagent and bacterially derived indoles. See G.  
3 Lombard and V. Dowell, *J. Clin. Microbiol.* (1983)  
4 18:609-613. The sensitivity of the reagent was  
5 compared to two other indole detecting compounds:  
6 Kovac's reagent and DMCA. Ehrlich's reagent was  
7 reported to be 10 times less sensitive than DMCA and  
8 10 times more sensitive than Kovac's reagent in  
9 detecting indole.

10

11 While Ehrlich's reagent has been used to roughly  
12 detect the presence of pyrroles or indoles in a  
13 targeted material, improved compositions and methods  
14 for detecting such heterocycles are desirable,  
15 especially methods that provide for detecting  
16 pyrrole-containing molecules that are markers of  
17 particular disease states.

18

#### 19 SUMMARY OF THE INVENTION

20

21 The present invention provides methods of assaying  
22 pyrrole-containing biological compounds.

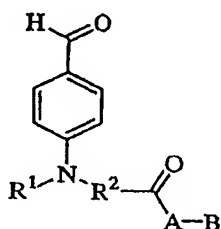
23 In one case the method involves:

- 24 1) contacting the biological compound with either:  
25 a) an optionally labelled derivatizing agent  
26 (bound to or able to bind to a solid  
27 support), wherein the derivatizing agent  
28 forms a reaction product with the  
29 biological compound (preferably via  
30 covalent attachment thereto), followed by

- 1 exposure to a detectable molecule which  
2 forms a complex with the reaction product;  
3 or  
4 b) an optionally labelled derivatizing agent  
5 not bound to a solid support, wherein the  
6 derivatizing agent forms a reaction product  
7 with the biological compound (preferably  
8 via covalent attachment thereto), followed  
9 by exposure to a binding agent specific to  
10 the biological compound in the reaction  
11 product, said binding agent being bound to  
12 a solid support; or  
13 c) a binding agent bound to a solid support,  
14 said binding agent being specific to the  
15 biological compound and forming a complex  
16 therewith, followed by exposure to an  
17 optionally labelled, derivatizing agent  
18 which forms a reaction product with the  
19 biological compound moiety of said complex  
20 (preferably via covalent attachment  
21 thereto); and  
22  
23 2) determining the amount of bound biological  
24 compound by detecting the detectable molecule,  
25 or by determining the amount of free or bound  
26 binding agent or by measuring the amount of  
27 label present.  
28

1 Preferably, the method of assaying pyrrole-containing  
2 biological compounds is Method 1, described in part  
3 a) above. Method 1 involves the following steps:

- 4  
5 1) contacting a biological compound with a  
6 derivatizing agent of the following structure in  
7 the bound form;



- 8  
9 wherein  $R^1$  is an alkyl group,  $R^2$  is an alkyl  
10 group, A is a linking group and B is a solid  
11 support, and wherein the contact induces  
12 formation of a reaction product, and wherein the  
13 reaction product comprises the covalent  
14 attachment of the biological compound to the  
15 derivatizing agent; followed by contacting the  
16 reaction product with a detectable molecule,  
17 wherein the contact induces specific binding of  
18 the detectable molecule to the reaction product  
19 to provide a complex; and  
20  
21 2) determining the amount of bound biological  
22 material by detecting the detectable molecule.  
23

1 Preferably the detectable molecule is a monoclonal  
2 antibody (MAb) specific to the biological compound.  
3 Preferably the solid support is a microtitre or a  
4 treated glass slide.

5

6 Preferably the method of assaying pyrrole-containing  
7 biological compounds is Method 2 described in part b)  
8 above. Method 2 involves the following steps:

9

- 10 1) contacting the biological compound with an  
11 optionally labelled derivatizing agent in  
12 solution to form a reaction product therewith  
13 (preferably via covalent attachment thereto)  
14 followed by exposure to a binding agent bound to  
15 a solid support, said binding agent being  
16 specific to the biological compound in the  
17 reaction product and
- 18  
19 2) determining the amount of bound biological  
20 compound by determining the amount of labelled  
21 derivatizing agent bound to the solid support.

22

23 Preferably the derivatizing agent is biotinylated  
24 Ehrlich's reagent. Preferably the solution  
25 containing the reaction product is neutralised prior  
26 to contact with the bound binding agent. Preferably  
27 the bound MAb is bound to a solid support, suitably a  
28 microtitre plate or a treated glass slide.

29

1 Preferably the derivatizing agent is labelled with a  
2 labeling molecule, suitably a radio-labelled,  
3 fluorescent label, enzyme label or the like.  
4 Preferably the amount of bound biological compound is  
5 determined by detecting the amount of labelled  
6 derivatizing agent bound on the solid support.

7  
8 Method 2 takes into account the fact that relatively  
9 strong acid conditions are required for the reaction  
10 of derivatizing reagents with pyrroles. Thus, most  
11 non-covalent interactions, such as antibody-antigen  
12 complexes, would be disrupted under these conditions.  
13 To overcome this problem, pyrrolic units in the  
14 biological sample are targeted in Method 2 by  
15 reaction in solution with derivatizing agent to form  
16 a reaction product, preferably via covalent  
17 attachment thereto followed by capture of the  
18 reaction product on a surface coated with specific  
19 antibodies.

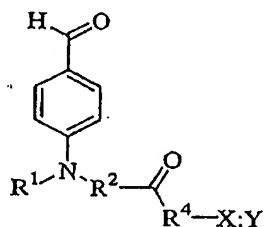
20  
21 Preferably, the method of assaying pyrrole-containing  
22 biological compounds is Method 3, described in part  
23 a) above. Method 3 involves the following steps:

24  
25 1) contacting a biological compound with a  
26 derivatizing agent in solution to form a  
27 reaction product wherein the derivatizing agent  
28 comprises a first partner of a strong binding  
29 pair.

- 1    2)    contacting the reaction product with a solid
- 2            support having a second partner of the strong
- 3            binding pair on its surface, to form a bound
- 4            complex with the reaction product;
- 5    3)    contacting the bound complex with a detectable
- 6            molecule;
- 7    4)    determining the amount of bound biological
- 8            compound by detecting the amount of detectable
- 9            molecule bound to the solid support.

10

11    Preferably the derivatizing agent is a *p*-  
 12    dimethylaminobenzaldehyde derivative, and in bound  
 13    form has the following structure:



14

15    wherein  $R^1$  is an alkyl group,  $R^2$  is an alkyl group,  $R^4$   
 16    is a heteroalkyl group, X is a first partner of a  
 17    strong binding pair and Y is a solid support having a  
 18    second partner of a strong binding pair on its  
 19    surface.

20

21    Preferably the solution containing the reaction  
 22    product is neutralized prior to contact with the  
 23    solid support.

24



1 In one embodiment the first partner of the strong  
2 binding pair is from avidin and the second partner of  
3 the strong binding pair is from biotin.

4 Alternatively the first partner of the strong binding  
5 pair is from biotin and the second partner of the  
6 strong binding pair is from avidin. In a second  
7 embodiment the first partner of the strong binding  
8 pair is from biotin and the second partner of the  
9 strong binding pair is from streptavidin.

10 Alternatively the first partner of the strong binding  
11 pair is from streptavidin and the second partner of  
12 the strong binding pair is from biotin.

13

14 Preferably the detectable molecule is a monoclonal  
15 antibody specific to the biological compound moiety  
16 of the complex. Suitably the solid support is a  
17 microtitre plate or a treated glass slide.

18

19 The present invention also provides a method of  
20 purifying an antigen, said method comprising;

21

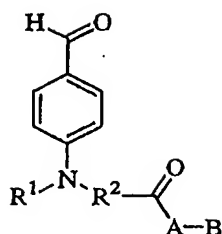
22 1) contacting a pyrrole-containing biological  
23 compound with one of;

24 a) an optionally labelled derivatizing agent  
25 (bound or able to bind to a solid support)  
26 wherein the derivatizing agent forms a  
27 reaction product with the biological  
28 compound (preferably via covalent  
29 attachment thereto) followed by exposure to

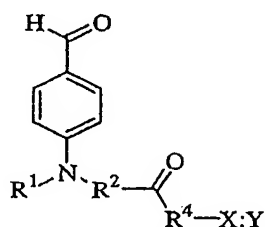
- 1 a detectable molecule which forms a complex  
2 with the reaction product; or  
3 b) an optionally labelled derivatizing agent,  
4 not bound to a solid support, wherein the  
5 derivatizing agent forms a reaction product  
6 with the biological compound (preferably  
7 via covalent attachment thereto), followed  
8 by exposure to a binding agent bound to a  
9 solid support wherein the binding agent is  
10 specific to a biological compound in the  
11 reaction product; or  
12 c) a binding agent bound to a solid support,  
13 said binding agent being specific to the  
14 biological compound, and forming a complex  
15 therewith, followed by exposure to an  
16 optionally labelled, derivatizing agent,  
17 which forms a reaction product with the  
18 biological compound moiety of said complex  
19 (preferably via covalent attachment  
20 thereto); and  
21 2) eluting the biological compound from the solid  
22 support.

23  
24 This method allows easy preparation of an antigen,  
25 which can then be used in screening for an antigen  
26 detection agent, for example antibody.

27  
28 Preferably the derivatizing agent for use in the  
29 method of purifying an antigen is of the following  
30 structure in bound form:



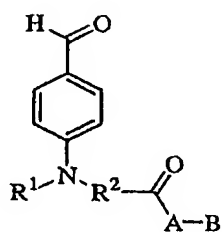
1  
 2 wherein  $\text{R}^1$  is an alkyl group,  $\text{R}^2$  is an alkyl group, A  
 3 is a linking group and B is a solid support.  
 4  
 5 Preferably the labeled derivatizing agent has the  
 6 following structure in bound form:



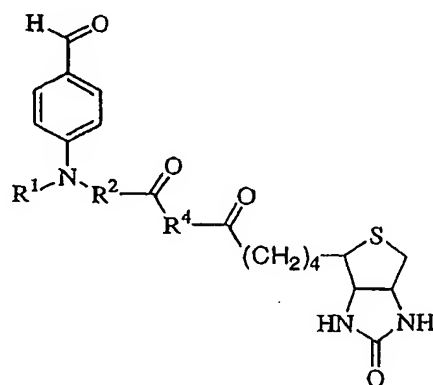
7 wherein  $\text{R}^1$  is an alkyl group,  $\text{R}^2$  is an alkyl group,  $\text{R}^4$   
 8 is a heteroalkyl group, X is a first partner of a  
 9 strong binding pair and Y is a solid support having a  
 10 second partner of a strong binding pair on its  
 11 surface.  
 12  
 13 Preferably the detectable molecule is a monoclonal  
 14 antibody specific to the biological compound.  
 15  
 16 Optionally the derivatizing agent is labelled with a  
 17 radio-label, fluorescent label, enzyme label or the  
 18 like.  
 19

1 The present invention also provides compounds for use  
 2 in the method of assaying pyrrole-containing  
 3 biological compounds.

4  
 5 In one case, the compound is of the following  
 6 structure:



7  
 8 wherein R<sup>1</sup> is an alkyl group, R<sup>2</sup> is an alkyl group, A  
 9 is a linking group and B is a solid support.  
 10  
 11 More preferably the labeled derivatizing agent has  
 12 the following structure:



1 wherein R<sup>1</sup> is a straight-chain alkyl group containing  
2 1 to 10 carbon atoms, R<sup>2</sup> is a straight-chain alkyl  
3 group containing 1 to 10 carbon atoms, and R<sup>4</sup> is a  
4 straight-chain heteroalkyl group containing 2 to 10  
5 carbon atoms and at least 2 heteroatoms.

6

#### 7 BRIEF DESCRIPTION OF THE DRAWINGS

8

9 FIGS. 1a-1i show mass spectrometry spectra of pyrrole  
10 crosslink-containing peptides.

11

12 FIG. 2 schematically represents Methods 1, 2 and 3.

13

14 FIG. 3 shows the difference of pyrrole capture of  
15 bone peptides at different dilutions.

16

17 FIG. 4 shows pyrrole capture at different dilution of  
18 biological sample using detection antibodies specific  
19 for isoaspartyl telopeptides.

20

21 FIG. 5 shows pyrrole capture assay for digested and  
22 immobilized collagen-containing tissues.

23

24 FIG. 6 shows the results for a serial dilution of  
25 biotin-ER reacted bone digest or a streptavidin  
26 coated plate detected with NTP monoclonal antibody.

27

28

29

## 1 DESCRIPTION OF THE SPECIFIC EMBODIMENTS

2

3 Introduction

4 The present invention provides methods of assaying  
5 pyrrole-containing biological compounds and chemical  
6 compositions that can be used in those methods. In  
7 Method 1 of the present invention, a biological  
8 sample, that may have been processed, is contacted  
9 with a solid support bound or able to bind  
10 derivatizing agent. Pyrrolic units in the biological  
11 sample react with the derivatizing agent, thereby  
12 immobilizing components containing the pyrroles on  
13 the solid support. The reacted solid support is  
14 contacted with a detectable molecule, such as a MAb,  
15 which interacts with a portion of the immobilized  
16 biological material. Detection of the detectable  
17 molecule on the solid support indicates that the  
18 biological material contains pyrrolic units.

19

20 In Method 2 of the present invention an optionally  
21 processed biological sample is contacted with a non-  
22 bound, optionally labeled derivatizing agent in  
23 solution. The derivatizing agent is suitably labelled  
24 with a radio-label, fluorescent label, enzyme label  
25 or the like. The derivatizing agent reacts with the  
26 pyrrolic units in the biological sample to form a  
27 reaction product wherein the reaction product  
28 comprises the covalent attachment of the derivatizing  
29 agent and the pyrrolic units in the biological

1 compound. The solution containing the reaction  
2 product is neutralised.

3

4 The reaction product may be contacted with a solid  
5 support bound MAb specific to the biological sample.  
6 The MAb reacts with the reaction product to form a  
7 complex immobilized on the solid support. Detection  
8 of the labeled molecule on the solid support  
9 indicates that the biological material contains  
10 pyrrolic units.

11

12 In method 3 of the present invention, an optionally  
13 processed biological compound is contacted with a  
14 derivatizing agent, wherein the derivatizing agent  
15 comprises a first binding partner of a strong binding  
16 pair, suitably from biotin. The derivatizing agent  
17 is in solution. Pyrrolic units in the biological  
18 compound react with the derivatizing agent to form a  
19 reaction complex. The solution containing the  
20 reaction product is neutralised prior to contact with  
21 a solid support coated with a second binding partner  
22 of the strong binding pair, to form a bound complex  
23 with the reaction product. Suitably the second  
24 binding partner is from streptavidin. The solid  
25 support is then contacted with a detectable molecule,  
26 preferably a MAb specific to the biological compound  
27 moiety of said complex. The amount of bound  
28 biological compound is determined.

29

30 FIG. 2 schematically illustrates Methods 1, 2 and 3.

1 Definitions

2 "Alkyl group" refers to a straight-chain, branched or  
3 cyclic group containing a carbon backbone and  
4 hydrogen. Examples of straight-chain alkyl groups  
5 include methyl, ethyl, propyl, butyl, pentyl and  
6 hexyl. Examples of branched alkyl groups include i-  
7 propyl, *sec*-butyl and t-butyl. Examples of cyclic  
8 alkyl groups include cyclobutyl, cyclopentyl and  
9 cyclohexyl. The "alkyl" group also refers to  
10 alkylene groups.

11

12 Alkyl groups are substituted or unsubstituted. In a  
13 substituted alkyl group, a hydrogen on the carbon  
14 backbone is replaced by a different type of atom  
15 (e.g., oxygen, nitrogen, sulfur, halogen). For  
16 instance, 2-hydroxyethyl is an ethyl group where one  
17 of the hydrogens is replaced by an OH group; 2-  
18 chloropropyl is a propyl group where one of the  
19 hydrogens is replaced by a Cl group.

20

21 "Heteroalkyl group" refers to a straight-chain,  
22 branched or cyclic group containing a carbon-  
23 heteroatom backbone and hydrogen. Heteroatoms  
24 include, without limitation, oxygen, nitrogen and  
25 sulfur. The following groups are examples of  
26 heteroalkyl groups:  $-\text{CH}_2\text{OCH}_2\text{CH}_3$ ,  $-\text{NH}(\text{CH}_2)_5\text{NH}-$  and  
27  $-\text{NH}(\text{CH}_2)_2\text{SS}(\text{CH}_2)_2\text{NHC}(\text{O})(\text{CH}_2)_5\text{NH}-$ . As with alkyl  
28 groups, heteroalkyl groups are substituted or  
29 unsubstituted.

30



1 "Leaving group" refers to a chemical group that is  
2 capable of being displaced in a nucleophilic  
3 substitution reaction. Examples of leaving groups  
4 include  $-Cl$ ,  $-Br$ ,  $-OC(O)CH_3$  and  $-SPh$ .  
5  
6 "Linking group" refers to a chemical group that  
7 connects one chemical group to another. For  
8 instance, in the compound  $CH_3C(O)-NH(CH_2)_5NH-CH_3$ , the  
9 group  $-NH(CH_2)_5NH-$  is a linking group between  $CH_3C(O)-$   
10 and  $-CH_3$ .  
11

#### 12 Types Of Biological Materials To Be Examined

13

14 The present method is used to determine the presence  
15 of pyrrolic units in biological materials, including  
16 pyrrolic crosslinks in collagen extracts. For some  
17 time, researchers have proposed that pyrrolic  
18 components exist in collagen. See Scott et al.,  
19 *Biosci. Rep.* (1981) 1:611-618; see also Kuypers et  
20 al., *Biochem. J.* (1992) 283:129-136. Only indirect  
21 support for the proposal has been available, however,  
22 as the isolation and characterization of collagen  
23 derived pyrrolic crosslinks has proven difficult.  
24

25 Experimental results presented herein provide direct  
26 confirmation of pyrrolic crosslinks in collagen. See  
27 Examples 4 and 5. A series of peptides from human  
28 bone collagen enzyme digests were isolated using a  
29 solid support bound *p*-aminobenzaldehyde, indicating

1 the presence of pyrrolic units in the collagen.  
2 Analysis of the isolated peptides using mass  
3 spectrometry showed that a relatively large number of  
4 the peptides possessed masses extremely close to the  
5 theoretic masses of complexes derivatized at  
6 predominantly the N-telopeptide sites of collagen.

7  
8 Pyrrolic crosslinks are particularly prevalent in  
9 bone collagen where they result from the natural  
10 maturation process of the tissue. During resorption  
11 of bone by osteoclasts, fragments of collagen  
12 crosslinked by pyrroles are released into the  
13 circulation. Their concentration in various  
14 biological fluids provides an indication of the rates  
15 of bone degradation. Increased bone resorption rates  
16 are associated with a number of diseases, including,  
17 for example, the following: osteoporosis, osteo- and  
18 rheumatoid arthritis, and diseases involving  
19 abnormalities of vitamin D or parathyroid hormone  
20 such as osteomalacia and hyperparathyroidism. By  
21 detecting pyrrolic crosslinks using the present  
22 invention, therefore, one is able to characterize and  
23 monitor such diseases.

24  
25 Another example of biological materials that can be  
26 assayed using the present invention is the  
27 isolevuglandins (e.g., levuglandin E<sub>2</sub>).  
28 Isolevuglandins are formed through free radical-  
29 mediated oxidation of polyunsaturated fatty acid  
30 esters in low-density lipoproteins. These compounds

1 react with various proteins to produce pyrroles in  
2 vivo. See Brame et al., *J. Biol. Chem.* (1999)  
3 274:13139-13146; see also Salomon et al., *J. Biol.*  
4 *Chem.* (1999) 274:20271-20280.  
5  
6 Free radical-mediated oxidation has been implicated  
7 in a wide variety of human diseases, including  
8 atherosclerosis, cancer and neurodegenerative  
9 diseases. See B. Halliwell and J. Gutteridge,  
10 *Methods Enzymol.* (1990) 186:1-85. Specifically, the  
11 oxidative modification of low density lipoproteins is  
12 a key step in atherosclerosis etiology. The  
13 detection of isolevuglandin derived pyrroles  
14 accordingly provides a method for diagnosing and  
15 monitoring atherosclerosis.  
16  
17 Proteins modified by non-enzymatic glycosylation  
18 reactions constitute a third example of a biological  
19 material that can be assayed using the present  
20 invention. Threose, primarily derived from the  
21 breakdown of ascorbate (vitamin C), represents one  
22 instance of this reaction. It is particularly  
23 reactive with lysine residues in proteins and forms  
24 pyrrolic structures (e.g., formyl threosyl pyrrole)  
25 as a result. See R. Nagaraj and V. Monnier, *Biochem.*  
26 *Biophys. Acta* (1995) 1253:75-84.  
27  
28 Detecting formyl threosyl pyrrole is specifically  
29 useful for monitoring patients with diabetes. It is  
30 also an example of an advanced glycation end-product

1 (AGE). AGEs are associated, for example, with  
2 abnormal neurofibrillar structures in Alzheimer's  
3 disease, and the presence of increases AGEs in  
4 lipoproteins appears to accelerate the oxidative  
5 reactions leading to atherosclerosis. Therefore, the  
6 detection of formyl threosyl pyrrole provides a  
7 method for diagnosing and monitoring those diseases  
8 as well.

9

#### 10 Methods Of Processing Biological Materials

11

12 Subject biological materials assayed using the  
13 present method may be unprocessed (e.g., urine, serum  
14 or plasma) or processed. A primary goal of  
15 processing is the solubilization of the sample.

16

17 Where the biological material is a tissue, it is  
18 usually de-fatted by two brief extractions (e.g., 15  
19 min.) with acetone or chloroform:methanol (2:1 v/v).  
20 Mineralized tissues are, for example, powdered  
21 underliquid nitrogen and subsequently demineralized  
22 using extraction with 0.5 M EDTA at pH 7.5 for 72-96  
23 hours at 4 °C. Connective tissue samples are  
24 typically denatured by heating the sample in saline  
25 at pH 7.4 for 30 min at 70 °C.

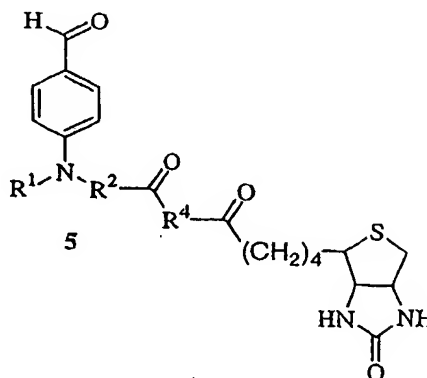
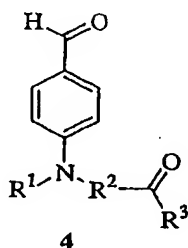
26

27 Sample solubilization typically involves the use of  
28 proteases rather than chemical hydrolysis, as  
29 pyrroles exhibit chemical instability under certain  
30 conditions. Where proteases are used, a sample is

1 treated with a suitable proteolytic enzyme (e.g.,  
 2 trypsin) at a suitable temperature (e.g., 37 °C).  
 3 Examples of other enzymes one can use to solubilize a  
 4 biological material include chymotrypsin, pronase,  
 5 pepsin, proteinase K and members of the cathepsin  
 6 family (B, L, N or K). For any chosen enzyme, one of  
 7 ordinary skill can readily determine a suitable  
 8 reaction buffer pH and temperature.

#### 10 Derivatizing Agents

12 The derivitizing agents used in the present assay are  
 13 p-amino benzaldehyde derivatives used in the present  
 14 assay are of the structures 4 and 5. R<sup>1</sup> in the  
 15 structures is an alkyl group; R<sup>2</sup> is an alkyl group;  
 16



17 R<sup>3</sup> is a hydroxyl group or leaving group; and, R<sup>4</sup> is a  
 18 heteroalkyl group.

20 The substituent R<sup>1</sup> is preferably a straight-chain  
 21 alkyl group containing 1 to 10 carbon atoms. It is  
 22 more preferably a straight-chain alkyl group

1 containing 1 to 5 carbon atoms. Most preferably,  $R^1$   
 2 contains 1 carbon atom (*i.e.*,  $-\text{CH}_3$ ).

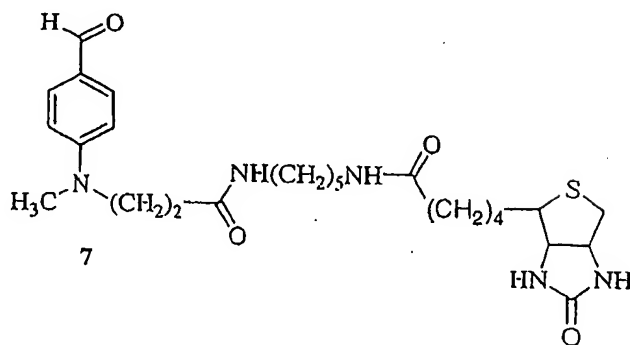
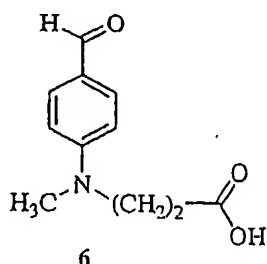
3  
 4 The substituent  $R^2$  is preferably a straight-chain  
 5 alkylene group containing 1 to 10 carbon atoms. It  
 6 is more preferably a straight-chain alkylene group  
 7 containing 1 to 5 carbon atoms. Most preferably  $R^2$   
 8 contains 2 carbon atoms (*i.e.*,  $-\text{CH}_2\text{CH}_2-$ ).

9 The substituent  $R^3$  is preferably  $-\text{OH}$ ,  $-\text{OR}^5$  (where  $R^5$   
 10 is a straight chain alkyl such as methyl),  $-\text{Cl}$  or  
 11  $\text{SR}^5$ . It is more preferably  $-\text{OH}$  or  $-\text{OR}^5$ . Most  
 12 preferably  $R^3$  is  $-\text{OH}$ .

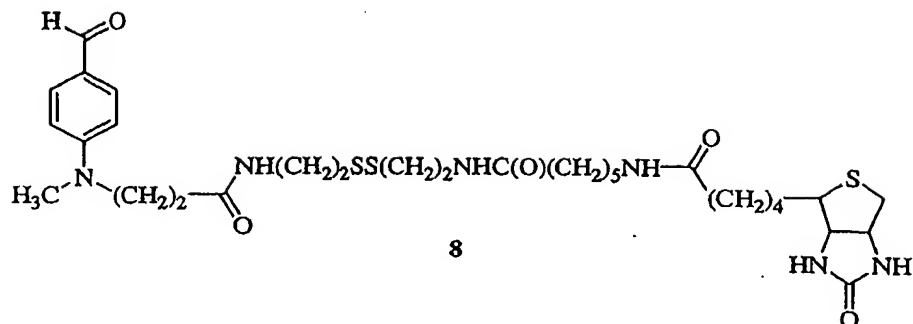
13  
 14 The substituent  $R^4$  is preferably a straight-chain  
 15 heteroalkyl group containing 2 to 10 carbon atoms and  
 16 at least 2 heteroatoms. It is more preferably a  
 17 straight-chain heteroalkyl group containing 4 to 10  
 18 carbon atoms and at least 2 nitrogen atoms. Most  
 19 preferably  $R^4$  is  $-\text{NHCH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}-$  or  
 20  $-\text{NHCH}_2\text{CH}_2\text{SSCH}_2\text{CH}_2\text{NHC(O)}-\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{CH}_2\text{N}-$ .

21  
 22 Examples of three preferred derivatizing agents are  
 23 *p*-amino benzaldehyde derivatives are shown as  
 24 compounds 6, 7 and 8:

25



1

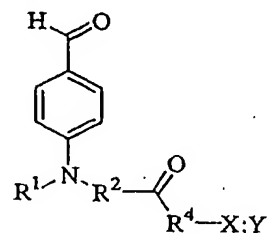
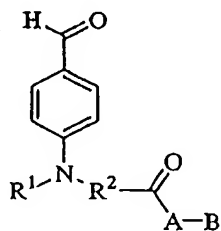


## 2 Modes Of Attachment To A Solid Support

3

4 The derivatizing agent is attached to the solid  
 5 support through either a covalent bond or a  
 6 noncovalent interaction. A derivatizing agent in  
 7 bound form attached to solid support through a  
 8 covalent bond is represented by compound 9; a  
 9 derivitizing agent in bound form attached to a solid  
 10 support through a noncovalent interaction is  
 11 represented by compound 10:

12



13 The substituents of compound 9 are defined as  
 14 follows: R<sup>1</sup> is an alkyl group; R<sup>2</sup> is an alkyl group;  
 15 A is a linking group and B is a solid support.  
 16 Preferably, R<sup>1</sup> and R<sup>2</sup> are alkyl groups containing 1  
 17 to 10 carbon atoms and A is a heteroalkyl group.

1 More preferably,  $R^1$  and  $R^2$  are alkyl groups  
2 containing 1 to 5 carbon atoms and A is a heteroalkyl  
3 group comprising at least 1 nitrogen atom. Most  
4 preferably,  $R^1$  is  $-\text{CH}_3$  and  $R^2$  is  $-\text{CH}_2\text{CH}_2-$ . The  
5 substituents of compound 10 are defined as follows:  
6  $R^1$  is an alkyl group;  $R^2$  is an alkyl group;  $R^4$  is a  
7 heteroalkyl group; X is a first partner of a strong  
8 binding pair and Y is a solid support having a second  
9 partner of a strong binding pair on its surface.  
10 Preferably,  $R^1$  and  $R^2$  are alkyl groups containing 1  
11 to 10 carbon atoms and  $R^4$  is a straight-chain  
12 heteroalkyl group containing 2 to 10 carbon atoms and  
13 at least 2 heteroatoms. More preferably,  $R^1$  and  $R^2$   
14 are alkyl groups containing 1 to 5 carbon atoms,  $R^4$   
15 is  $-\text{NH}(\text{CH}_2)_5\text{NH}-$  or  $-\text{NH}(\text{CH}_2)_2\text{SS}(\text{CH}_2)_2\text{NHC}(\text{O})(\text{CH}_2)_5\text{NH}-$ .  
16 Most preferably,  $R^1$  is  $-\text{CH}_3$  and  $R^2$  is  $-\text{CH}_2\text{CH}_2-$ .  
17  
18 Where a covalent bond is used for attachment, a  
19 surface is typically derivatized to afford a reactive  
20 functional group such as an alcohol or amine. For  
21 instance, compound 6 is coupled to a Nunc Covalink™  
22 plate, available from Nalge Nunc International,  
23 through the formation of an amide bond with a C8-  
24 primary amine. See [www.nalgenunc.com](http://www.nalgenunc.com). A second  
25 example of a suitable solid support is a DNA-BIND™  
26 surface, available from Corning. See  
27 [www.scienceproducts.corning.com](http://www.scienceproducts.corning.com). One reacts a  
28 bifunctional compound, such as 1,5-diaminopentane,  
29 with the surface to provide available amine groups



1 for covalent attachment. A compound such as 6, which  
2 contains a carboxylic acid, is coupled to the surface  
3 groups through the formation of an amide bond. A  
4 third example of a solid support is a glass  
5 substrate. A glass slide is treated with  
6 aminopropyl-triethoxysilane to provide a glass  
7 substrate containing a reactive amine across its  
8 surface. See U.S. 5,919,523. The derivatized slide  
9 is reacted with compound such as 6 in the presence of  
10 a suitable reagent that induces amide bond formation.  
11 Where a noncovalent interaction is used for  
12 attachment, a compound containing one partner of a  
13 strong binding pair is adhered or bonded to the solid  
14 support. The other partner of the pair is covalently  
15 attached to a derivatizing agent to form a conjugate.  
16 When the conjugate is contacted with the solid  
17 support, a strong interaction (e.g., one or more  
18 hydrogen bonds) immobilizes the conjugate on the  
19 support.  
20  
21 An example of a strong binding pair is a  
22 biotin:avidin complex. (A biotin:streptavidin  
23 complex is another example.) Typically, a support  
24 surface is derivatized to include biotin or avidin.  
25 Avidin coated polystyrene plates (i.e., Reacti-Bind™  
26 NeutrAvidin™ coated plates) are available, for  
27 instance, from Pierce. See [www.piercenet.com](http://www.piercenet.com). The  
28 avidin coated plate is contacted with a biotin  
29 containing *p*-aminobenzaldehyde derivative such as  
30 compound 7. The resulting biotin-avidin complex

1 serves to attach compound 7 to the solid support  
2 through noncovalent interactions.

3

#### 4 Examples Of Different Assay Formats

5

6 The method of assaying pyrrole-containing biological  
7 compounds is typically run in a multi-well plate  
8 (e.g., 96-well plate), but other assay formats are  
9 also used. The method is also performed using a  
10 strip format, where a derivatizing agent is  
11 immobilized on the strip surface. A third exemplary  
12 format involves the use of a polymeric bead (e.g.,  
13 polystyrene bead) on which a derivatizing agent is  
14 immobilized. Yet another format involves the use of  
15 micro-array or chip technology; use with surface  
16 plasmon resonance technology.

17

#### 18 Contact Of Extract/Isolate With Detection Compound

19

20 To perform a method of the present invention, a  
21 biological fluid or processed biological material is  
22 contacted with a solid support bound derivatizing  
23 agent. The biological material may be solubilized in  
24 a suitable solvent to form a solution prior to the  
25 contact. When a multi-well format is used, for  
26 example, the solution and any additional elements  
27 readily discernable to one of ordinary skill in the  
28 art is added to one or more wells. For the strip  
29 format, a strip is dipped into a solution containing  
30 the biological material; and, for the bead format, a

1 vial or tube is used to mix the beads and the  
2 solution.

3

4 Regardless of assay format, contact between a  
5 pyrrole-containing biological material and the  
6 support bound derivatizing agent induces a coupling  
7 reaction. The result of the reaction is a covalent  
8 bond between the biological material and the  
9 derivatizing agent. This serves to immobilize the  
10 pyrrole-containing biological material on the solid  
11 support.

12

13 When desired, the solid support bound biological  
14 material is washed with at least one suitable solvent  
15 to remove impurities from the reaction medium. The  
16 solid support is typically dried after a washing  
17 step. A variety of drying techniques are used,  
18 including air drying, drying under reduced pressure  
19 and thermal drying.

20

#### 21 Methods Of Detection Using A Detectable Molecule

22

23 In a method of the present invention, the immobilized  
24 material is contacted with a detectable molecule.  
25 The detectable molecule specifically binds to a  
26 portion of a targeted biological material. If the  
27 material on the solid support is not the targeted  
28 material, the detectable molecule will not bind to it  
29 with high affinity.

30

1 The detectable molecule can bind to the targeted  
2 biological material through either covalent or  
3 noncovalent bonds. Typically, the detectable  
4 molecule is a polyclonal, monoclonal or phase  
5 library-derived antibody that binds to the biological  
6 material through noncovalent bonds. Preferably, it  
7 is a monoclonal antibody.

8  
9 The detectable molecule is typically detectable in  
10 one of three ways: 1) it contains functionality one  
11 can observe; 2) it induces a chemical reaction that  
12 produces an observable product; or 3) it interacts  
13 with a second molecule that either contains  
14 functionality one can observe or induces a chemical  
15 reaction that produces an observable product.  
16 Functionality one can observe includes chemical  
17 groups that exhibit a measurable effect upon  
18 stimulation. For instance, the following chemical  
19 groups exhibit such an effect: a chemical group that  
20 absorbs light at a certain wavelength (a chromophore)  
21 and a chemical group that fluoresces upon exposure to  
22 a particular wavelength of light. A chemical  
23 reaction that produces an observable product  
24 includes, for example, a reaction producing a  
25 fluorescent compound, a luminescent compound or a  
26 chromophoric compound.

27  
28 Where the targeted biological material is collagen  
29 derived pyrrole crosslinks, an example of a  
30 detectable molecule is a monoclonal antibody (NTP)

1 raised against a synthetic octapeptide comprising  
2 part of the sequence of the  $\alpha 2(I)$  N-terminal  
3 telopeptide. The NTP antibody is contacted with the  
4 immobilized biological material. A secondary  
5 antibody (goat anti-mouse IgG-peroxidase conjugate)  
6 is introduced; which interacts with a portion of the  
7 NTP antibody. Upon addition of 3,3',5,5'-  
8 tetramethyl-benzidine dihydrochloride and hydrogen  
9 peroxide, a chromophoric compound exhibiting an  
10 absorbance at 450 nm is produced. See Example 6.

11

#### 12 Contact of Extract/Isolate with Detection Compound

13 To perform Method 2 or 3 of the present invention, a  
14 biological fluid or processed biological material is  
15 contacted with a labeled derivatizing agent in  
16 solution. The derivatizing agent is labeled with a  
17 labeling molecule. Any suitable solvent as known by  
18 a person skilled in the art may be used. A coupling  
19 reaction between pyrrole-containing biological  
20 material results in a reaction product comprising the  
21 derivatizing agent covalently bonded to any pyrrole-  
22 containing biological material.

23

#### 24 Methods of Detection Using a MAb

25 In Method 2 of the present invention the reaction  
26 product is immobilised by contact of the solution  
27 with a MAb bound on a solid support.

28

29

30

1 Example 1: Preparation of compound 6.  
2 N-Methyl-N-cyanoethyl-4-amino benzaldehyde (available  
3 from Enterwin Chemicals, China or Sigma-Aldrich, USA)  
4 (150 mg) was dissolved in 7.5 M NaOH, 6% H<sub>2</sub>O<sub>2</sub> (5 ml)  
5 and refluxed for 2 hours. The hydrolysate was  
6 acidified by addition of concentrated HCl, dried  
7 under vacuum and redissolved in ethanol (1.5 ml). An  
8 aliquot of the solution (1 ml) was added to 0.2 M  
9 NaOH (1 ml) and applied to an anion exchange column  
10 (Bio-Rad AG 1-X8; 2 ml, pretreated with 2 M HCl, 2 M  
11 NaOH and equilibrated with water). The column was  
12 washed with water (12 ml) before elution of the bound  
13 material with 2 M HCl. The eluent was dried under  
14 vacuum and the residue resuspended in water (1 ml).  
15 A small amount of residue (soluble in ethanol but  
16 containing no compound 6) was removed after which the  
17 aqueous fraction was dried under vacuum (yielding 7  
18 mg of material) and redissolved in 0.1%  
19 trifluoroacetic acid (1 ml). Aliquots (100 µl) of  
20 the material was chromatographed on a Waters RCM  
21 Prep-Pak® C<sub>18</sub> column (25 mm x 100 mm, 10 µm) pumped  
22 at 4 ml/min. The buffers used were 0.1% TFA (buffer  
23 A) and 70% acetonitrile, 0.1% TFA (buffer B) with a  
24 gradient of 5 minutes at 5% B followed by a linear  
25 increase to 70% B over 35 minutes. Monitoring at 330  
26 nm showed a single major peak which eluted at 28.3  
27 min. Fractions corresponding to the peak were pooled  
28 and dried under vacuum (yield = 3 mg). Analysis of  
29 the material by electrospray mass-spectrometry in  
30 negative-ion mode using a MAT 900 mass spectrometer

1 (Finnigan MAT, Bremen, Germany) revealed the major  
2 ion as  $[M-H] = 206.2$  which corresponds to the  
3 expected value for N-methyl-N-propionic acid-4-amino  
4 benzaldehyde  $M_r$  207.2.

5

6 Example 2: Preparation of compound 7.

7 Compound 6 (3 mg) was dissolved in water (3 ml) and  
8 biotin-pentyl amine (30 mg; Pierce) was added. A  
9 solution of 1-ethyl-3-(3-dimethylamino-  
10 propyl)carbodiimide/N-hydroxysuccinimide (0.035  
11 M/0.028 M respectively; 3 ml) was added and heated to  
12 50 °C for 4 h. The resulting solution was dried  
13 under vacuum and chromatographed using the  
14 preparative RCM Prep-Pak® column described in  
15 Example 1. The gradient applied was 20% B for 5 min  
16 followed by a linear increase to 60% B over 30 min.  
17 Two major components were detected, one eluting at 15  
18 min. (unreacted acid) and one eluting at 18 min. The  
19 component eluting at 18 min was analyzed by positive-  
20 ion electrospray mass-spectrometry and showed  $[M+H]$   
21 of 518.7 and  $[M+Na]$  of 540.6. These values  
22 corresponded to the calculated  $M_r$  of compound 7 of  
23 517.7. Compound 7 reacted with pyrrole carboxylic  
24 acid in 4 M HCl to give a characteristic pink color  
25 absorbing at 573 nm.

26

27 Example 3: Preparation of compound 8.

28 Compound 6 (1 mg) was dissolved in 0.1 M MES buffer  
29 pH 5 (1 ml) and a ten-fold molar excess of cystamine  
30 ( $H_2N(CH_2)_2SS(CH_2)_2NH_2$ ) was added. The solution pH was

1 adjusted to 5 using HCl, and a solution of 1-ethyl-3-  
2 (3-dimethylamino-propyl)-carbodiimide/N-  
3 hydroxysuccinimide (0.035 M/0.028 M respectively; 1  
4 ml) was added. The solution was heated to 50 °C for  
5 4 h. The resulting aminated derivative was purified  
6 by HPLC, eluting with 10 mM TFA and an acetonitrile  
7 gradient (monitoring 330 nm). Biotinylation of the  
8 aminated derivative was performed using succinimide-  
9 LC-biotin (Pierce) according to the manufacturer's  
10 instructions and again purified by HPLC. Structure 8  
11 was confirmed by MALDI-TOF mass spectrometry.

12

13 Example 4: Reaction of compound 7 with a bone  
14 digest.

15 De-fatted human bone (7 g) was powdered in a Spex  
16 freezer-mill in liquid nitrogen. The resultant  
17 powder was decalcified by 3 x 2-day extractions in  
18 0.5 M EDTA, pH 8 at 4 °C, washed with water and  
19 lyophilized. The decalcified bone powder (1.1 g) was  
20 suspended in 0.1 M citrate buffer, pH 5, heated to  
21 70°C for 1 hour to denature the triple-helical  
22 structure and allowed to cool to 45 °C. Papain (100  
23 U) was added, and the digest was incubated for 4  
24 hours. The pH of the digest was adjusted to 7.4 by  
25 the addition of 1 M Tris, and the temperature was  
26 lowered to 37 °C for an overnight digestion with  
27 protease type X (100 U). The completed digest  
28 (estimated as 110 µM collagen by total pyridinium



1 crosslink content) was frozen, lyophilized and  
2 suspended in water (7 ml).

3

4 After the addition of compound 7 (50 µg) to the bone  
5 digest (500 µl), the mixture was acidified by the  
6 addition of 12 M HCl (250 µl). During incubation for  
7 30 min at room temperature, the solution turned  
8 cherry-pink in color, and spectrometry showed the  
9 presence of an absorption maximum at 571.7 nm  
10 (characteristic of product from reaction of 4-  
11 dimethylamino benzaldehyde with pyrrole). The acid  
12 was neutralized by the addition of 12 M NaOH (approx.  
13 220 µl) followed by 40 mM phosphate buffer (20 ml).

14

15 Example 5: Isolation of conjugation product between  
16 compound 7 and pyrrolic peptides.

17 A monomeric avidin column (5 ml) was prepared  
18 according to manufacturer's (Pierce) instructions.  
19 The reacted bone digest of Example 4 at neutral pH  
20 was added slowly to the column, which was then washed  
21 with 6 column volumes of PBS followed by 1 column  
22 volume of water. The biotinylated material was  
23 eluted at about 1 ml/min with 1 M acetic acid  
24 adjusted to pH 2.5 with ammonia, and 8 fractions (5  
25 ml) were collected.

26

27 *Estimation of biotinylated compounds by competitive*  
28 *ELISA.* In order to assess the efficiency of the  
29 monomeric avidin column (Example 5), a competitive

1 ELISA was developed. Immulon 4 immunoassay plates  
2 were coated with streptavidin (25 nM) in<sup>b</sup> PBS for 2  
3 hours at 37 °C. Samples or standards in PBS 0.1%  
4 Tween, 0.5% fat-free milk powder (FFMP; 110 µl) were  
5 added to biotinylated peroxidase (Sigma; 10 ng/ml;  
6 110 µl) in PBS Tween, 0.5% FFMP in a U-bottomed 96-  
7 well plate. The mixed samples were transferred to  
8 the washed, streptavidin-coated plate and incubated  
9 for 90 min at 37 °C. After washing the plate 3 times  
10 with PBS/0.1% Tween, the peroxidase substrate (200  
11 µl) tetramethyl-benzidine dihydrochloride (TMB) was  
12 added (0.1 mg/ml) in 0.05 M citrate/phosphate buffer  
13 pH 5, 0.012% v/v hydrogen peroxide. The reaction was  
14 stopped by the addition of 3 M sulphuric acid (50 µl)  
15 after 15 min.

16  
17 *Analysis of isolated material by HPLC.* Material  
18 eluted from the avidin column was reduced in volume  
19 (100 µl) and chromatographed on a reversed phase HPLC  
20 column (4.6 x 100 mm; C<sub>18</sub>; particle size 3 µm). The  
21 column was equilibrated with 0.1% TFA (buffer A), and  
22 peptides were eluted over 35 min with linear  
23 gradients formed with 70% acetonitrile, 0.1% TFA  
24 (buffer B). The eluent was monitored at 214 nm, 280  
25 nm and at 330 nm. Each fraction from the HPLC was  
26 dried and redissolved in water (2 µl). An aliquot (1  
27 µl) was mixed with α-cyano-4-hydroxy-cinnamic acid (1  
28 µl of a 10 mg/ml solution in 70% acetonitrile 0.1%  
29 TFA), dried onto a sample plate and analyzed by

1 MALDI-TOF mass spectrometry (Voyager-DE; Applied  
2 Biosystems) calibrated externally using bradykinin.  
3  
4 The MALDI-TOF mass spectrometry spectra of each  
5 fraction is shown in FIG. 1. As there were  
6 insufficient quantities of many of the smaller  
7 peptides to obtain amino acid composition data, some  
8 ambiguities in their structural assignments did  
9 arise. In particular, the mass difference between  
10 Glu and Ile/Leu is equivalent to an additional  
11 hydroxyl group and, for the isolated peptide with  $M_r$   
12 = 1086 (FIG. 1a), the ambiguity is due to the  
13 possible presence of a hydroxylated crosslink. Thus,  
14 this peptide may contain Gly and Glu (from either the  
15 C- or N-telopeptides of the  $\alpha 1$  chain) or, for a  
16 hydroxylated crosslink, a Gly residue linked with  
17 either Ile (from the  $\alpha 1$  helix) or a leucine (from the  
18  $\alpha 2$  helix). Even where the amino acid composition is  
19 known, the precise location of the residues may not  
20 be clear, as in the case of the peptide with  $M_r$  = 957  
21 (FIG. 1a) containing the biotinylated pyrrole with a  
22 single Gly residue. This residue is shown in a  
23 helical position (which could be at the N- or C-  
24 terminal overlap sites) but could also be derived  
25 from the  $\alpha 2(I)$  N-telopeptide: this peak may contain  
26 a mixture of Gly-containing peptides from the  
27 different locations. The  $M_r$  = 1029 peptides shown in  
28 FIG. 1e and 1g could have the same alternatives of  
29 glutamate or hydroxylated pyrrole-leucine/isoleucine.

1 The peaks corresponding to a loss of Gly (FIG. 1b,  
2 1c) are probably losses due to the energy of the  
3 laser-desorption rather than discrete peptides, but  
4 these peaks provide additional evidence for the  
5 peptide structures proposed. The structures of the  
6 larger peptides shown in the other panels are  
7 unambiguous.

8

9 Example 6: Detection of pyrrole crosslinks (Method 1)

10 The carboxyl-Ehrlich derivative was coupled to a Nunc  
11 Covalink® plate via a C8-primary amine group. After  
12 adding the derivative to the plate (250 pmole/well in  
13 100 µl MES buffer, pH 4.5) followed by 100 µl of 1-  
14 ethyl-3-(3-dimethylamino- propyl)carbodiimide/ N-  
15 hydroxysuccinimide (0.035M / 0.028M respectively),  
16 the plate was heated to 50°C and left overnight at  
17 room temperature. The plate was aspirated and washed  
18 with 4M HCl and 3 times with water. Each well  
19 coupled the equivalent of 66 pmoles of the reagent  
20 and the coupling was confirmed using HPLC.

21

22 Samples (110µl), prepared in a separate plate, were  
23 acidified by the addition of 8M HCl (110 µl). The  
24 acidified samples (200 µl) were then added to the  
25 Ehrlich reactive plate and agitated for 1 hour at  
26 room temperature. The plate was aspirated and washed  
27 3 times in 4 M HCl, 3 times in water and finally 3  
28 times in PBS/0.1% Tween; 10mM lysine, 0.5% fat-free  
29 milk powder (assay buffer). The antibodies used were

1 a monoclonal antibody (NTP) raised against the  $\alpha 2(I)$   
2 telopeptide (1:1000 dilution) or affinity-purified,  
3 polyclonal antibodies raised against the isoaspartyl  
4  $\alpha 2(I)$  telopeptide (1:250 dilution). After incubation  
5 for 17 hours at 4 °C, the plate was washed 3 times  
6 with PBS-Tween and incubated for 1 hour with  
7 secondary antibodies, goat anti-mouse IgG-peroxidase  
8 conjugate, used at a dilution of 1:4000. The plate  
9 was washed 3 times with PBS-Tween, and 200  $\mu$ l of  
10 peroxidase substrate, 3,3',5,5'-tetramethyl-benzidine  
11 dihydrochloride (TMB) is added (0.1 mg/ml) in 0.05 M  
12 citrate/phosphate buffer, pH 5, containing 0.012% v/v  
13 hydrogen peroxide. The reaction is stopped by the  
14 addition of 3 M sulfuric acid (50  $\mu$ l), and the  
15 absorbance was measured at 450 nm using a Dynatech MR  
16 7000 plate reader.

17

18 Using the pyrrole-capture assay, serial dilutions of  
19 a bone digest (starting at ~1.0 nmole/well collagen)  
20 reacted in the Ehrlich plate gave progressively  
21 decreasing reactivity with NTP antibody (FIG. 3). At  
22 a fixed concentration (0.125 nmole/well) of pyrrole-  
23 crosslinked bone peptides on the plate, preincubation  
24 of the NTP antibody with serial dilutions adolescent-  
25 human urine gave essentially complete inhibition of  
26 colour development.

27

28 When pyrrole crosslink-containing peptides in urine  
29 from an adolescent were reacted with the plate, the

1 NTP antibody failed to detect any telopeptide (FIG.  
2 4). A possible explanation for this is that the  
3 large quantities of non-isomerised telopeptide found  
4 in urine at this age may not be extensively  
5 crosslinked. This is supported by the fact that the  
6 polyclonal antibody raised against the isoaspartyl  
7 rearranged peptide did show reactivity towards  
8 captured peptides in urine from an older subject (30  
9 years), see FIG. 4.

10

11 The specificity of the assay was demonstrated by  
12 showing that peptides derived from cartilage and  
13 skin, which have no pyrrolic crosslinks, gave very  
14 little reaction in the assay compared to the bone  
15 digest and a phorphobilinogen standard (FIG. 5).

16

17 Example 7: Detection of pyrrole-containing peptides  
18 from enzyme digests of bone (Method 2)

19 A tryptic digest of demineralized human bone (0.5 ml  
20 containing approximately 5  $\mu$ M collagen) was reacted  
21 with biotinylated Ehrlich's reagent (50  $\mu$ g; 0.1  
22  $\mu$ moles) in 3M HCl for 30 min at room temperature. The  
23 sample was neutralized by the addition of 2M NaOH and  
24 diluted to 10 ml in phosphate buffered saline, pH 7.5  
25 (PBS) containing 0.1% Tween 20. Serial (x2)  
26 dilutions of this pre-reacted mixture were prepared  
27 in PBS-Tween for addition to the detection plate.

28

29 The detection microtitre plate was coated with a  
30 monoclonal antibody (NTP) recognizing an octapeptide

1 sequence containing the cross-linking region of the  
2 N-telopeptide of collagen type I  $\alpha 2$  chain. In order  
3 to gain the appropriate orientation of the antibody,  
4 the plate was initially coated (3 hours at room  
5 temperature) with anti-mouse IgG (raised in donkey)  
6 by adding to each well 0.2 ml of a solution  
7 containing 1  $\mu$ g/ml protein in PBS. After washing 3  
8 times with PBS-0.05% Tween 20, the NTP antibody (1  
9  $\mu$ g/ml in PBS) was added and reaction allowed to  
10 proceed for 1 hour at room temperature. The plate  
11 was again washed 3 times with PBS-Tween.

12

13 Serial dilutions of the pre-reacted mixture were  
14 added to the coated plate and incubated at room  
15 temperature for 2 hours. The plate was washed 3  
16 times with PBS-Tween and the biotin-pyrrole detected  
17 by the addition of streptavidin-horseradish  
18 peroxidase (Amersham plc, Little Chalfont, UK)  
19 diluted 1:2000 in PBS-Tween. After 1 hour the plate  
20 was washed 3 times in PBS-Tween and the colour  
21 developed by the addition of 200  $\mu$ l of peroxidase  
22 substrate, 3,3',5,5'-tetramethyl-benzidine  
23 dihydrochloride (TMB) is added (0.1 mg/ml) in 0.05 M  
24 citrate/phosphate buffer, pH 5, containing 0.012% v/v  
25 hydrogen peroxide. The reaction is stopped by the  
26 addition of 3 M sulfuric acid (50  $\mu$ l), and the  
27 absorbance was measured at 450 nm using a Dynatech MR  
28 7000 plate reader.

29

1 Example 8: Detection of pyrrole-containing peptides  
2 from enzyme digests of bone (Method 3)

3 Biotinylated Ehrlich's reagent was reacted with  
4 tryptic peptides of human bone collagen as described  
5 for Method 2.

6  
7 For the detection plate, high-binding microtitre  
8 plates (Immunlon 4) were coated with streptavidin (1  
9 µg/ml in PBS) by incubating for 3 hours at 37°C. The  
10 plates were washed 3 times with PBS-Tween and any  
11 remaining binding sites were blocked by incubation at  
12 room temperature for 1 hour with 3% bovine serum  
13 albumin in PBS-Tween. The plate was again washed 3  
14 times with PBS-Tween. Alternatively, ready coated  
15 plates are available commercially from several  
16 sources, such as Streptavidin-coated Combiplates from  
17 Thermo Labsystems, Basingstoke, UK.

18  
19 Serial dilutions of the pre-reacted mixture were  
20 added to the streptavidin-coated plate and incubated  
21 at room temperature for 2 hours. The plate was  
22 washed 3 times with PBS-Tween and, after the addition  
23 of NTP monoclonal antibody (1:1000 dilution in PBS-  
24 Tween), the plate was incubated at 4°C for 18 hours.  
25 The plate was washed 3 times with PBS-Tween and  
26 incubated for 1 hour with secondary antibodies, goat  
27 anti-mouse IgG-peroxidase conjugate, used at a  
28 dilution of 1:4000. After washing the plate 3 times  
29 with PBS-Tween, colour development with TMB and



1 recording optical densities at 450 nm using the plate  
2 reader were done as described previously.

3

4 Example 9: Preparation of pyrrole containing antigens  
5 from bone collagen peptides

6 Peptides were prepared from powdered, decalcified  
7 human bone by digestion with cathepsin K. The bone  
8 (10mg) was suspended in 1.0ml of 50mM sodium acetate  
9 buffer, pH 5.0, containing 2mM EDTA and 2mM  
10 dithiothreitol and, after the addition of 0.1mg  
11 recombinant cathepsin K dissolved in 100 $\mu$ l PBS,  
12 digestion was continued for 24 hours at 37°C with  
13 gentle agitation. The digest was centrifuged  
14 (13,000g) to remove any undigested tissue, and the  
15 supernatant solution was desalted on a column (1.0 x  
16 12cm) of Sephadex G25 equilibrated and eluted with  
17 0.2M acetic acid. Pooled fractions containing the  
18 bone peptides were lyophilised and reacted with  
19 biotinylated, disulphide Ehrlich's reagent (compound  
20 8; 0.1mg; 0.2 $\mu$ moles) in 3M HCl at room temperature  
21 for 30 mins. The solution was neutralized by the  
22 addition of 2 M NaOH and diluted to 10ml with PBS.

23

24 The bone digest Ehrlich conjugate was applied to a  
25 5ml column of immobilized avidin (Pierce Chemical Co)  
26 prepared according to the manufacturer's  
27 instructions, and the column washed with PBS  
28 containing 10 mM dithiothreitol and located by  
29 monitoring the column effluent at 230nm. Pooled  
30 fractions were dialysed against PBS to remove

- 1 reducing agent. This material was mixed with an
- 2 equal volume of adjuvant and used directly for
- 3 immunization of rabbits and mice.